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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 3/20, B01D 15/08, G01N 30/48

A1

(11) International Publication Number: WO 94/15951

(43) International Publication Date: 21 July 1994 (21.07.94)

(21) International Application Number: PCT/SE94/00016

(22) International Filing Date: 13 January 1994 (13.01.94)

(30) Priority Data:
9300090-9 14 January 1993 (14.01.93) SE

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: AFFINITY PURIFICATION BY THE USE OF A COMPLEXED LIGAND

(57) Abstract

Method for affinity based isolation of a first target substance, said method comprising: (i) contacting said first target substance with a matrix to which is linked a ligand having affinity for said first target substance; (ii) eluting said first target substance. The method is characterized by the ligand having been initially complexed/bound to a second target substance having weaker binding ability to the ligand than the first target substance and providing multi-point attachment to the matrix by carrying several ligand binding sites and/or other matrix binding sites on its molecule enabling adsorption of the first target substance during step (i) without the second target substance becoming eluted from the matrix. In one mode the second target substance is thermo-reactive, and steps (i) and (ii) are run at different temperatures separated by the cloud point of the second target substance.

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AFFINITY PURIFICATION BY THE USE OF A COMPLEXED LIGAND

Technical field

The use of biological recognition as a tool for the separation of proteins constitutes the basis for affinity chromatography and other affinity based purification procedures. The recognition utilized is between a ligand and a target substance, the latter being the substance to be isolated. Encountered target substances often are of protein/polypeptide, lipid, carbohydrate or nucleotide structure. The ligand may be a substrate analogue or an inhibitor (in case the target substance is an enzyme), an antibody, a carbohydrate etc.

Generally methods for affinity based isolation of a target substance (T_1) from a mixture of substances containing said target substance comprises the steps of

(i) contacting said mixture in an aqueous medium with a matrix to which is linked a ligand, for instance a group specific ligand, having binding affinity for said target substance (T_1) under conditions causing said target substance to bind to said ligand, and

(ii) changing the conditions offered by said medium so that said target substance (T_1) will elute from said matrix,

The matrix is normally fitted for chromatography and thus insoluble in the aqueous medium that preferably is water with or without a certain content of a water-miscible co-solvent.

In most cases the procedures taking advantage of affinity based purification have placed the affinity step as late as possible in the protocol after that the most difficult substances have been removed. During recent years there has been a tendency towards employing affinity steps during the earlier part of the protocol. In these situations a great part of the undesired substances may be removed with a significant reduction of volumes. In order to accomplish this one has tended to use ligands which are high-resolving with respect to the substance to be isolated, the reason being that high resolving ligands often are substrates for enzymes present

during the early steps and therefore easily are degraded. Group specific ligands, such as textile dyes, hydrophobic ligands, chelating agents etc, have been used. Group specific ligands have resulted in isolation rather than purification in the affinity step. After an affinity step utilizing a group specific affinity ligand, one may want to further purify the substance, but now in a considerably smaller sample volume and with fewer other proteins present. Group specific ligands have in addition had a tendency to cause several non-specific interactions which lead to binding of unwanted proteins to the ligand. Normally such proteins can be removed by washing before release of the target substance to be isolated.

Prior art

Coating of adsorbents with hydrophilic polymers has previously been used in chromatography of biomolecules for imparting new properties to the matrix, e.g., formation of anion exchange phases by cross-linking of polyimines with bifunctional glycidoxy compounds (Alpert et al., J. Chromatogr. 185 (1980) pp 37), or for decreasing non-specific interactions between protein and the matrix (Santarelli et al., J. Chromatogr. 443 (1988) pp 55 (dextran coating); and Letot et al., J. Liq. Chromatogr. 4 (1981) pp 1311 (poly(N-vinyl pyrrolidone) coating).

During the priority year some preliminary results with the inventive method have been published (Galaev et al., J. Chromatogr. 648 (1993) pp 367).

Temperature-responsive polymers have earlier been attempted for modifying binding and elution of a target substance (Gewehr et al., Macromol. Chem. 193 (1993) pp 249). Gel permeation chromatography was carried out using porous glass modified with a temperature-responsive polymer (poly(N-isopropylacryl amide). The elution of dextrans with various molecular masses was strongly affected by the temperature due to a change in the effective pore size of the matrix. This can be attributed largely to the transition of polymer molecules from coils to globules on the surface of the glass beads.

Ethylene glycol (30%) has been used in the eluent in order to accomplish elution by a temperature shift (Peuch et al., FEBS Letters 87 (1978) pp 232).

Problems solved by the invention and objectives

In the field of affinity based separation methods there is a general desire for methods facilitating improved resolution, purity, specificity, reduction of non-specific interactions (in particular involving the ligand), elution of target substances (T_1) under milder conditions etc. The objective of the present invention is to provide this type of improvements in the methods given in the introductory part.

The invention

According to the inventive concept these objectives are achieved by the ligand being initially complexed/bound to a second target substance (T_2) having weaker binding ability to the ligand than the first target substance (T_1) and providing multi-point attachment to the matrix by carrying two or more ligand binding sites and/or other matrix binding sites on its molecule enabling adsorption of the first target substance (T_1) without the second target substance (T_2) becoming eluted from the matrix.

In case the sample, in addition to T_1, also contains other substances T_x binding to the ligand, the second target substance (T_2) runs the risk of being displaced and washed away from the matrix when the sample containing the first target substance (T_1) is applied to the matrix. T_1 as well as T_x runs an apparent risk of being adsorbed together, which will make the selective isolation of T_1 difficult. In order to prevent this, T_2 should have an affinity for the ligand that is lower than the affinity of T_1 but higher than each of the affinities of T_x and a plurality of sites binding to ligand molecules and/or the matrix.

The second target substance (T_2) may exhibit a plurality of polar and/or non-polar and/or polarizable groups in order to provide the multi-point binding to ligands and/or to the matrix. Potential groups that may be present in T_2 are amide, ester, keto, aromatic rings, nitro, thioether, ether, alcohol,

mercapto, sulfo, saturated and unsaturated hydrocarbon residures etc. It is believed that T_2 preferably should be non-ionic. Our present results indicate successful isolation procedures according to the invention for T_2 that is soluble in the solution utilized during the adsorption step. However, since the results in fact have been achieved with forms of T_2 that have been insolubilized by adsorption to the used matrix, one also must emphasize that also insoluble forms of T_2 will function.

In order to provide multi-point attachment T_2 may be a polymer, preferably synthetic, that may be a homo- or copolymer. In case T_2 is a synthetic polymer, one and the same selected functional group is regularly repeated within the T_2 molecule. Synthetic polymer shall be interpreted to also encompass native biopolymers that have been chemically modified to contain several sites for attachment to the ligand.

Particularly preferred T_2 substances have been found among polymers containing a plurality of identical amide groups such as in polymerized vinyl compounds in which the vinyl group is N-substituted on a cyclic amide or imide, for instance pyrrolidone or caprolactam rings.

T_2 may be a heterobifunctional structure or substance, for instance a biomolecule, exhibiting a low affinity for the ligand as one functionality and some second functionality for attaching the heterobifunctional structure in place on the matrix. Said second functionality is then selected to be independent of the experimental conditions employed in the affinity part of the isolation procedure. The second functionality, for instance, may provide hydrophobic binding to the matrix.

The two target substances T_1 and T_2 may exhibit the same structure with a weaker binding ability for T_2 being accomplished by chemical coupling, for instance to a polymer or other structure that causes retainment of the structure to the affinity matrix (heterobifunctional target substances). In this latter case the binding to the ligand is caused by the structure that is common to T_1 and T_2. In case T_1 and T_2 are oligomers built up of the same monomers, a weaker affinity of T_2 may be caused by a lower oligomeri. The pair of target

molecules (T_1 and T_2) may also be two different proteins reacting with the same ligand (epitope) but with different affinities, for instance cross-reacting antibodies or proteins which are recognized to displace each other in displacement chromatography separation. In case of mono-binding proteins the one (T_2) with the weaker affinity has been modified (aggregated, polymerized) so as to exhibit several binding sites for the ligand.

The ligand may contain structures of the same type as suggested for T_2, although there are several known ligands that in addition also have ionic structures. In particular ligands containing chromophores with conjugated double or triple bonds and condensed aromatic rings have found use as group specific ligands (Cibacron Blue, Procion Scarlet and other textile dyes).

The ligand may be attached by covalent links or physical adsorbtion to the matrix, the main point being that it shall remain on the matrix at least during steps (i) and (ii).

In general the fitness of the second target substance (T_2) will depend on the structure of the ligand employed and the target substance (T_1) to be isolated. In order to check the fitness of a potential combination, the binding constants and number of binding sites of T_1 and T_2, respectively, relative the ligand can be determined as outlined in Example 4.

Pretreatment of the matrix with the second target substance (T_2) may be accomplished simply by contacting the matrix with an aqueous solution containing excess of T_2 and the appropriate buffer (the excess is in relation to the matrix bound ligand). Afterwards the matrix is washed to remove excess of T_2.

The loading of sample (step (i)) is performed in conventional manner, for instance by applying sample to break-through of the substance to be isolated. See the experimental part.

Also the elution step (ii) may be performed in the conventional manner for each respective ligand, for instance by passing an eluent causing dissociation of the complex formed in step (i) between the ligand and T_1. The present invention can provide milder elution conditions, for instance by applying eluents having lower concentrations of salt, without decreasing yield and quality of the target substance (T_1) to be isolated.

In one aspect of the invention elution may simply be accomplished by changing the temperature. This requires a low affinity target substance (T_2) that is temperature-responsive (thermo-reactive) in the sense that it has a temperature (cloud point) below which its water solution is clear and above which the same solution becomes cloudy (transition from soluble to insoluble forms). In this mode, step (i) is performed at one temperature followed by a change in temperature in step (ii) such that the cloud point is passed. If one selects to increase or decrease the temperature is likely to depend on the type of T_2 used, structure of ligand etc. According to knowledge obtained at the filing date of this patent application the adsorption (step (i)) can preferably be performed at a temperature above the cloud point and the elution step (step (ii) by lowering the temperature so that elution takes place at a temperature below the cloud point.

In order to accomplish elution by a shift in temperature according to this part of the inventive concept, T_2 should preferably have a cloud point that is lower than the temperature at which the substance to be isolated is irreversibly changed or damaged, for instance for proteins below the irreversible denaturation temperature. In case normal proteins are to be isolated the cloud point often is below 45°C. The use of water as eluent or as a sample constituent sets a lower limit for cloud points of T_2 at about 0°C (freezing point). In short this means that for biotechnological applications, for instance most isolation of substances exhibiting polypeptide, carbohydrate, nucleotide and lipid structure and isolations utilizing proteinic ligands, T_2 ought to be selected among those having a cloud point within the temperature range of 0-45°C. These values of cloud points refer to values measured in the solution used for adsorption/elution.

The cloud point of an aqueous polymer solution is determined as the temperature at which the soultion becomes turbid on heating. The value of the cloud point depends slightly on the polymer concentration, the minimal cloud point value being referred to as the lower critical temperature.

Temperature-responsive water-soluble polymers, non-ionic surfactants, and hydrogels as reagents in biotechnology have

recently being reviewed (Galaev et al., Enzyme Microb. Technol. 15 (1993) pp 354). Potential candidates to be used in temperature dependent elution may be selected among those discussed therein.

We have found poly(N-vinyl caprolactam) to be particularly useful in combination with chromophoric ligands such as Cibacron Blue.

The inventive concept may, in principle, be applied to column or membrane chromatography, batch-wise procedures and any other isolation procedures utilizing a solid phase matrix carrying an affinity ligand. If one so prefers, steps (i) and (ii) may be performed in different modes, for instance a column mode may be selected for step (i) and a batch-wise mode for step (ii) and vice versa.

The modes of the invention considered to be most preferred at the filing date of this application are given in the experimental part.

EXPERIMENTAL

CHEMICALS:

Lactate dehydrogenase (LDH) type XXX-S from porcine muscle, β-NADH grade III, Cibacron Blue 3GA and PVP-40 with an average molecular mass of 40 000 and PVP-10 with an average molecular mass of 10 000 were purchased from Sigma (St Louis, MO, USA), Oxamic acid was purchased from BDH. N-vinyl caprolactam was purchased from Polysciences Inc. (Warrington, PA, USA). Polyethylene glycol (PEG) with molecular mass 20 000, polyvinylalcohol (PVA) with molecular mass 13 000, methylcellulose 15, hydroxyethyl cellulose 20, dextran F70 were purchased from Serva (Heidelberg, Germany).

The binding of Cibacron Blue to Sepharose® was carried out according to Heyns et al (Biochim. Biophys. Acta 358 (1974) pp 1) by linking Cibacron Blue to Sepharose CL-4B (Pharmacia Biotechnology Group, Sweden). The Cibacron Blue content in the final gel was 0.14 μmol per ml swollen gel (determined according Chambers (Anal. Biochem. 83 (1977) pp 551)).

Sepharose® CL-4B (Pharmacia Biotechnology group, Sweden) carrying Procion Scarlet H-2G as affinity ligand was obtained as a generous gift from prof. R.K. Scopes (Centre for Protein

and Enzyme technology, La Trobe University, Australia). The ligand had been linked to the gel according to Scopes (Anal. Biochem. 83 (1977) pp 551).

Poly(N-vinyl caprolactam) (PVCL) was synthesized by radical bulk polymerization according to Solomon et al (J. Appl. Polym. Sci. 12 (1968) pp 1843). The polymer obtained had a molecular mass of about 40 000 and a cloud point of 38°C in 20 mM Tris-HCl buffer (pH 7.3) in the presence of 0.1 M KCl.

Crude extract containing LDH: Minced pork was purchased in a local shop and homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.3) containing 1 mM EDTA (10 ml of buffer per gram of muscle tissue) The homogenate was filtered through a synthetic fibre pad to remove larger particulate matter, centrifuged for 15 minutes to remove cell debris and the supernatant was filtered through Munktell filter-paper to remove traces of fat. The porcine muscle extract was kept frozen without any loss of LDH activity and was applied directly, after thawing and filtering, to the column containing Cibacron Blue 3GA linked to Sepharose® CL-4B. The crude extract had an activity of 64 U/ml and a protein content of 5.5 mg/ml (specific activity 11,6 U/mg protein).

Crude extract containing secondary alcohol deydrogenase (SADH): The obligate anaerobic thermophilic organism **Thermoanaerobium brockii** was cultured in batch (Zeikus et al., Arch. Microbiol. 122 (1979) pp 41). Cells were harvested by centrifugation and stored frozen at -18°C. Cell were not maintained under strictly anaerobic conditions during harvesting and storage. Extraction of cells was carried out by sonication in 20 mM morpholinopropanesulphonate buffer (pH 6.5) containing 30 mM NaCl and 2 mM $MgCl_2$ (MES buffer)(4 ml per gram wet mass of cells). Cell debris was removed by centrifugation and the supernatant was applied directly to the column. The crude extract had an activity of 49 U/ml and a protein content of 14.0 mg/ml (specific activity 3.5 U/mg protein).

ANALYTICAL AND OTHER METHODS USED IN SEVERAL EXAMPLES

LDH was dialysed before use.

LDH activity was measured in the collected fractions according to Worthington Enzymes and Related Biochemicals (Freehold, N.J. (1982) 109-10).

The concentration of PVP was measured as absorption of polymer iodine complex at 480 nm, the complex being produced according to European Pharmacopoeia (Part 1, Maisonneuve S.A., Sainte-Ruffine, 2nd ed. (1986) 685-92)

Protein contents of collected fractions was monitored as absorbance at 280 nm.

The purity of different enzyme preparations was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemli (Nature 227 (1970) pp 680) using soybean trypsin inhibitor (MW 21 500), carbonic anhydrase (MW 31 000), ovalbumin (Mw 45 000) bovine serum albumin (MW 66 200) and phosphorylase B (MW 97 400) as standards.

EXAMPLE 1

The use of polyvinylpyrrolidone for blocking binding sites on Cibacron Blue bound to Sepharose® CL-4B (Pharmacia Biotechnology Group, Sweden) against unspecific binding of proteins.

General discussion:

The adsorbent used was an agarose gel matrix (Sepharose® CL-4B) to which Cibacron Blue is linked. Polyvinylpyrrolidone (PVP) has affinity for Cibacron Blue. Treatment of the adsorbent with PVP results in a gel which exhibits a significantly lowered non-specific binding of proteins. The treated gel will retain adsorption of proteins specifically binding to the Cibacron Blue, for instance lactate dehydrogenase (LDH). Retainment of the specific binding is likely to depend on the fact that the binding to individual ligand molecules is lower for PVP than for the specifically binding proteins. This in turn will cause PVP to be locally replaced by such proteins.

Analysis of the eluate from a PVP treated column to which LDH has been adsorbed indicates that no PVP is eluted from the column.

Experimental conditions:

LDH was adsorbed to PVP-shielded or PVP-untreated Blue Sepharose® packed in a column (0.9 x 1.8 cm I.D). Treatment with PVP was carried out by pumping an aqueous solution of PVP (1% (w/w), Mw 40 000) through the column packed with Blue Sepharose® which was then washed with 1.5 M KCl until no PVP could be detected in the eluate. To each of the two columns were then applied 120 U of LDH in a volume of 1.6 ml followed by elution with a flow of 0.16 ml/min and collection of fractions under 20 minutes. In separate runs the enzyme (LDH) was eluted with 1.5 M KCl (unspecific elution) or with a solution containing 10 mM oxamate and 0.1 mM NADH (specific elution).

Table I gives the LDH activity in units (U) for each fraction.

EXAMPLE 2

Purification of (a) LDH from crude homogenate of porcine muscle and (b) SADH from a crude extract of T. brockii by affinity chromatography on Sepharose® CL-4B to which Cibacron Blue or Procion Scarlet H-2G is linked as affinity ligand (PVP treated and untreated gels).

Chromatographic conditions:

Sepharose® CL-4B containing Cibacron Blue 3GA: All chromatographic experiments were carried out at room temperature using a 9.8 x 0.9 cm I.D. column at a flow rate of 0.55 ml/min. All solutions introduced to the column were 20 mM in Tris-HCl buffer (pH 7.3). The porcine muscle extract was applied to the column until breakthrough of LDH (120-200 ml with the untreated column and 60-70 ml with the PVP-shielded column). The column was washed with buffer until no more protein was detected in the eluate. Non-specific elution of LDH was performed with 1.5 M KCl and specific elution was performed with 10 mM oxamate plus 0.1 mM NADH. Fractions were collected every 20 min when eluted from the untreated column and every 5 min when eluted from the PVP-shielded (treated) column. The height equivalent to a theoretical plate (HETP) was calculated as HETP = L/N, where L is the bed length and N is the number of

theoretical plates calculated as $N = 5.54(V/W_{0.5})^2$, where V is the elution volume and $W_{0.5}$ is the peak width at half-height.

Sepharose® CL-4B containing Procion Scarlet H-2G: All chromatographic experiments were carried out at room temperature using a 2.8 x 0.9 cm I.D. column at a flow rate of 0.09 ml/min. For SADH elution all solutions introduced into the column were in MES buffer. The cell extract was applied to the column until breakthrough of SADH. The column was washed with buffer until no more protein was detected in the eluate. Non-specific elution of SADH was performed with 1.5 M KCl and specific elution with 0.5 mM NADP. For LDH elution all solution introduced into the column were in 20 mM Tris-HCl buffer (7.3). The porcine muscle extract was applied to the column until breakthrough of LDH. The column was washed with buffer until no more protein eluted. Non-specific elution of LDH was performed with 1.5 M KCl and specific elution with 10 mM oxamate + 0.1 mM NADH. Fractions were collected every 15 min.

The PVP-shielding (treatment) of the gels was performed with 1 % (w/w) PVP-40 000 solution followed by washing with 1.5 M KCl (pH 3.4) until no PVP was detected in the effluent. This was followed by re-equilibration of the column with an appropriate buffer.

Results and discussion:

Application until breakthrough of porcine muscle extract to Sepharose® CL-4B carrying Cibacron Blue 3GA resulted in binding of LDH along with significant amounts of foreign proteins, which could not be eluted with the buffer. Non-specific elution with 1.5 M KCl resulted first in the elution of foreign proteins followed by LDH with a recovery of 76 % as judged from activity judgements (Table II). The LDH recovery increased gradually to nearly 100 % only after subsequent purification cycles with application of porcine muscle extract to the same column. Thus, pretreatment of the gel with the homogenate resulted in masking of sites capable of non-specific irreversible binding of LDH. The same effect occurred during the specific elution with 0.1 mM NADH + 10 mM oxamate, but in contrast to non-specific elution foreign proteins adsorbed on the column were not eluted during specific elution. After

specific elution the column needed to be regenerated; foreign proteins could be eluted with 1.5 M KCl.

The PVP-treatment of the column resulted in a significant decrease in binding of foreign proteins and in an improved effectiveness of elution of LDH, either specifically or non-specifically. The decreased binding to the PVP-shielded gel eliminated the need for a regeneration step after the specific elution. The column could be used repeatedly after re-equilibration with buffer. The LDH recovery was about 100% even during the first run on a fresh column containing the PVP treated gel. Thus, PVP blocked the sites to which LDH irreversibly bound. Some proteins from the homogenate played the same role during the first application of porcine muscle extract to the column. No polymer was detected in the eluate from the PVP-protected column during chromatography of LDH (the sensitivity of the method of PVP assay being 0.1 mg/ml).

PVP treatment resulted in a significant improvement in the effectiveness of the elution. The HETP for the untreated column was 1.3 cm (non-specific elution) and 0.47 (specific elution). PVP-treatment decreased the HETP to 0.13 cm for both types of elution. LDH is eluted from the PVP-treated gel column as a symmetrical peak and about 95% of eluted enzyme can be collected in a volume of 8 ml. LDH is eluted from untreated gel column of the same size with significant tailing, and 95% of eluted LDH can be collected only in a volume of about 100 ml.

PVP, owing to its high affinity to Cibacron Blue 3GA, occupied sites capable of non-specific binding of foreign proteins and of irreversible binding of LDH and prevented this binding. LDH is considered to bind the PVP-treated Cibacron Blue gel only due to specific interactions between the Cibacron Blue ligand and the nucleotide binding site of the enzyme. The number of sites suitable for specific binding in the PVP-shielded gel is less than the total number of specific and non-specific LDH-binding sites in the untreated gel. This resulted in a decrease in dynamic capacity from 1150-1950 U/ml swollen gel (230-400 U/mmol Cibacron Blue ligand) for the untreated gel to 680-570 U/ml swollen gel (120-140 U/mmol Cibacron Blue ligand) for the PVP-shielded gel.

Table II shows virtually the same purity of LDH samples, obtained as the result of specific and non-specific elution from untreated and treated Cibacron Blue 3GA gels. In all cases, one step purification using dye-affinity absorption resulted in highly purified preparations.

Application of a **Thermoanaerobium brockii** extract to a column containing the Procion Scarlet ligand resulted in adsorption of SADH along with significant amounts of foreign proteins. This behaviour resembled that of LDH binding to the Cibacron Blue ligand, but in contrast to the latter case foreign proteins were eluted simultaneously with SADH by 1.5 M KCl, resulting in poor purification (Table III). Specific elution with 0.5 mM NADP resulted in a more pure enzyme preparation with a recovery of 70%, and the remaining 30% of SADH activity could be eluted together with other adsorbed proteins by 1.5 M KCl as a crude preparation (Table III). The specific elution at a lower loading gave lower recoveries and in the zonal mode SADH was not eluted by 0.5 mM NADP at all.

PVP treatment of Sepharose® CL-4B carrying Procion Scarlet as ligand resulted in an improvement in the effectiveness of specific elution and purity of the enzyme preparation, the recovery being 93%. Again, as with Cibacron Blue, PVP prevented adsorption of foreign proteins and non-specific interaction of SADH with the matrix. The decreased adsorption of foreign proteins resulted in a reasonable purity of SADH even after non-specific elution from the PVP-treated column (Table III and SDS-PAGE). The dynamic capacity of the column was decreased 4.3-10.5-fold after PVP-protection.

The non-specific interactions seemed to be more pronounced with gels carrying Procion Scarlet, which bound more foreign proteins than the gel carrying Cibacron Blue did, especially when porcine muscle extract was applied. LDH and SADH are eluted readily from the gel containing Procion Scarlet with 1.5 M KCl along with foreign proteins. One could see a deep red ring of haeme-containing proteins on the gel during KCl elution after application of the porcine muscle extract, and the LDH-containing eluate fractions were coloured red. A high adsorption of foreign proteins resulted in poor purity in LDH preparations after affinity chromatography on the gel

containing Procion Scarlet. Even in that case, PVP treatment improved the purity of the LDH preparations, although they had a lower specific activity compared with LDH preparations obtained after elution from untreated and PVP-treated gels carrying Cibacron Blue.

EXAMPLE 3

Adsorption and elution behaviour of LDH on Cibacron Blue affinity gel pretreated with poly(N-vinyl caprolactam). Thermal-induced displacement.

Experimentals:

The gel was Sepharose® CL-4B to which Cibacron Blue had been covalently linked. All chromatographic experiments were done using a thermostated 1.3 x 0.9 cm I.D. column. The flow rates were 0.55 ml/min (model experiments with pure LDH) and 0.17 ml/min (LDH extracts from minced porcine muscles). All LDH containing solutions introduced to the column were in 20 mM Tris-HCl buffer, pH 7.3. The elution of LDH was performed with 0.1 or 1.5 M KCl. Elution by the shift of temperature was typically carried out as follows: The sample was applied at 40°C followed by the elution with 0.1 M KCl at the same temperature (in the case of pure LDH to show that the enzyme was not eluted at these conditions or in the case of the porcine muscle extract to wash out foreign proteins). Then the pump was stopped, the column cooled to 23°C and the elution continued at this temperature with the same buffer that in some experiments was replaced with 1.5 M KCl. Temperature changes for pure LDH preparations were 30°C → 23°C, 35°C → 23°C, 40°C → 23°C and 45°C → 23°C.

The porcine muscle extract was applied to the column until break-through of LDH. The column was then washed with 0.1 M KCl in buffer until no more protein was detected in the elute, whereupon the elution was stopped, the temperature was lowered to 23°C and elution with 0.1 M KCl restarted.

Fractions were collected every 10 min (model experiments with pure LDH) and every 5 min (porcine muscle extracts).

PVCL-treatment (shielding) was performed with 1 % polymer solution followed by washing with 1.5 M KCl (pH 3.4) and re-equilibration of the column with an appropriate buffer.

The complex formation of PVCL with Cibacron Blue in solution was determined as in example 4.

The inhibition constants of LDH by Cibacron Blue were determined at 25°C and 40°C in 20 mM Tris-HCl (pH 7.3) in the presence and in the absence of 0.1 M KCl.

Results and discussion:

Elution profiles (with 0.1 M KCl followed by 1.5 M KCl) of pure LDH from untreated and PVCL-treated gel at room temperature (approx. 23°C) and 40°C. The elution profiles at room temperature and 40°C for the untreated gel were quite similar suggesting that this temperature shift had only a minor effect on the behaviour of the untreated column. Elution of LDH did not occur until the KCl concentration was increased to 1.5 M. This behaviour for the untreated gel was in complete agreement with the temperature effect on the binding of Cibacron Blue to LDH, which was estimated using the competitive inhibition constants. Addition of 0.1 M KCl increased the inhibition constant (hence a decreased efficiency of binding) from 0.1 μM to 0.65 μM at 23°C and from 0.14 μM to 0.76 μM at 40°C. This small decrease in binding efficiency caused by 0.1 M KCl was not enough to elute LDH from the untreated Cibacron Blue gel at both room temperature and 40°C. In contrast to the untreated gel, the PVCL-shielded gel behaved completely different. PVCL-shielding resulted in sharpened elution profiles at 40°C, an observation in line with the results presented in Examples 1-2. Most important, however, was the temperature response of the profiles. While LDH was not eluted at all from the untreated gel with 0.1 M KCl at 40°C, the enzyme was easily eluted with the same eluent from the PVCL-shielded gel already at room temperature. Elution of LDH at 40°C did not occur until the KCl concentration was increased to 1.5 M.

Elution profiles of LDH from PVCL-shielded column 0.1 M KCl at different temperatures. LDH is readily eluted with 0.1 M KCl at room temperature, rising the temperature hindered the elution, and practically no enzyme was eluted at 40 and 45°C, the temperatures above the cloud point of PVCL. Cooling down the column to temperatures below the cloud point resulted in LDH elution with the same buffer. The buffer containing 0.1 M

KCl is a much milder eluent thus resulting in broader LDH elution profiles from the polymer shielded gel compared with those obtained by elution with 1.5 M KCl.

It is conceivable that PVCL binds to the matrix via multi-point interaction with Cibacron Blue ligands. In solution one polymer molecule of PVCL bound efficiently 7-8 molecules of Cibacron Blue with a binding constant of 1.8 μM. The binding was independent of temperature below the cloud point of PVCL where the polymer is in the form of loose coils. When PVCL bound to Cibacron Blue molecules attached to the matrix, PVCL molecules could efficiently compete with LDH for the dye ligand. Even the small reduction in LDH binding efficiency caused by addition of 0.1 M KCl is enough to elute the enzyme from the PVCL-shielded column at room temperature. The strong multi-point attachment of PVCL to the matrix protects against complete washing out of the polymer from the column. The PVCL-shielded gel was used repeatedly for about 10 runs without loosing its temperature responsive property. Above the transition temperature (cloud point) PVCL molecules form compact globules striving for aggregation and formation of a separate phase. The polymer shielding becomes less tight and the dye ligand becomes more available for both specific and non-specific interactions with LDH. The elution with 0.1 M KCl in that case was inefficient, a more robust eluent was required. Above the transition temperature (cloud point) the behaviour of the PVCL-shielded gel was more similar to the untreated one, though some effect of the polymer resulted in a more sharp elution profile. Cooling of the PVCL-shielded column to which LDH is adsorbed results in the transition of the immobilized polymer molecules from compact globules to rather flexible coils. Due to their relative flexibility these coils could interact with more ligands compared with compact polymer globules. This interaction of PVCL molecules is efficient enough to displace LDH molecules bound to the affinity ligand. Thus cooling of the column resulted in the displacement of adsorbed LDH by immobilized PVCL polymer molecules and hence in complete elution of the adsorbed LDH enzyme without any changes in buffer composition.

The temperature sensitivity of polymer-shielded gels was used for the development of a ligand-affinity system where temperature was the only variable to be changed keeping the buffer composition constant. Crude porcine muscle extract was applied to a column at 40°C until break-through, the foreign proteins were washed out with 0.1 M KCl at 40°C, the flow interrupted, the column was cooled down to room temperature, and finally LDH was eluted with the same buffer. The chromatographic procedure resulted in virtually homogeneous LDH with a purification factor of 17 and an enzyme recovery of 90%.

EXAMPLE 4

Determination of binding constants between Cibacron Blue 3GA and different proteins.

The simplest model for independent binding of n Cibacron Blue molecules to the polymer was used, and it was treated according to a slightly modified procedure (Mayes et al, Biotech. Bioeng. 40 (1983) pp 639). The observed absorbance, A, can be described mathematically by

$$A = E_{CB}[CB] + E_X[X], \qquad (1)$$

where E_{CB} and E_X are molecular absorptives, [CB] and [X] are concentrations of uncomplexed and complexed Cibacron Blue, respectively

In the absence of polymer, the absorbance A' can be written as

$$A' = E_{CB}[CB]_0 \qquad (2)$$

where the subscript "0" indicates total concentration.

Also $$[CB] + [X] = [CB]_0 \qquad (3)$$

The change in absorbance ΔA due to the complexation of Cibacron Blue with the polymer may be written as

$$\Delta A = A - A' = (E_X - E_{CB})[X] \qquad (4)$$

E_{CB} was determined by direct measurement of Cibacron Blue absorbance, and E_X was determined by measurement of Cibacron Blue absorbance in the presence of excess of polymer.

The complex formation may be described as

$$[CB] + [\text{binding sites}] \text{ ---> } [X]$$

where the [binding sites] is the concentration of free binding sites on the polymer molecule. The equilibrium constant taken as the dissociation constant will be

$$K = [CB][\text{binding sites}]/[X] \quad (5)$$

The simplest assumption of independent binding of n Cibacron Bleu molecules to one polymer molecule corresponds to

$$[\text{binding sites}]_0 = n[\text{polymer}]_0 \quad (6)$$

Since $[\text{binding sites}] + [X] = [\text{binding sites}]_0$

Substituting (3) and (6) in (5) results in

$$K = ([CB]_0 - [X])([CB]_0 - [X])/[X]$$

Solving Equation (5) for [X] gives the physically significant root

$$[X] = 0.5([CB]_0 + K + n[\text{polymer}]_0) - \{([CB]_0 + K + n[\text{polymer}]_0)^2 - 4n[CB]_0[\text{polymer}]_0\} \quad (7)$$

The experimental data obtained for [X] from equation (5) over a range of polymer concentrations were fitted to the equation (7) allowing best fit values of the parameters K and n.

The spectral titration was performed at room temperature according to Mayes et al (Biotech. Bioeng. 40 (1983) pp 639). Sample and reference cuvettes each containing a solution of approximately 8 mM of Cibacron Blue in 50 mM Tris-HCl, pH 8.0, were placed in double beam Spectrophotometer Shimadzu UV-260. Small volumes (1-10 µl) of polymer solution (in case of PVP 5 % (w/w)) were added to the sample cuvette and equal volumes of buffer were added to the reference cuvette. The content of the cuvettes were mixed and the spectra in the region of 400 - 800 nm were registered. The experiments were performed at 0.1 M and 1.5 M KCl.

Using a difference spectra technique we studied the interaction of Cibacron Blue with different non-ionic, water-soluble polymers. No appreciable interaction of the dye with dextran, polyacrylamide, hydroxyethyl cellulose, and PEG was observed. A very weak interaction of hydrophobic type was detected for methylcellulose. Only PVP and PVCL and less efficiently PVA interacted with the dye.

The binding constant for PVP complexing with Cibacron Blue in solution was calculated as 2.2 µM and 1.8 µM, respectively. The PVP molecule with a molecular mass of 40 000 contained 30 sites capable of binding Cibacron Blue ligands. For PVP with a molecular mass of 10 000 the calculated value of the binding constant was 6.1 µM and the number of binding sites per polymer.

molecule was 8. Note should be taken that the calculated dependence of complex concentration on polymer concentration was very sensitive to the number of binding sites per polymer molecule.

The dye ligand was bound by a polymer segment with the molecular mass of 1000 - 1300 irrespective of the size of the PVP molecule.

Though the type of the difference spectra was changed with the increase of ionic strength, the binding constant of PVP-40 with Cibacron Blue was relatively insensitive to high ionic strength. In the presence of 1.5 M KCl, which is frequently used as non-specific eluent in the dye affinity chromatography, the binding constant was 5.7 μM, the binding sites being practically the same, 32.

In contrast to PVP, the interaction with PVA was significantly weaker. The calculated value of the binding constant was 16.7 μM and the number of binding sites per molecule was only 1.4. A polymer segment of PVA with molecular mass of about 10 000 complexed one Cibacron Blue molecule, ten times more, than in the case of PVP. PVA with a molecular mass of 13 000 interacted with the dye nearly stoichiometrically. This polymer could not provide multi-point attachment when interacting with a matrix carrying Cibacron Blue, and for this reason it could not be used for polymer shielding of a Cibacron Blue ligand according to the present invention.

Table IV presents our found values of binding constants for complexing of Cibacron Blue to several substances and corresponding values for some proteins determined by others. Proteins having lower binding constants than PVP bind stronger to Cibacron Blue, which also indicates that they potentially may be purified according to the invention.

In selecting suitable substances (T_2) that are to be used in combination with a ligand, possibly in order to isolate other substances (T_1), corresponding binding constants are determined and compared. In case a certain T_2 substance provides multi-point attachment and a higher binding constant to a ligand compared to the corresponding constant for the substance (T_1) to be isolated, this T_2-substance may potentially be used according to the invention. The final determination of the

fitness of a certain combination of a ligand with a T_2 substance, however, requires running the complete isolation procedure of the invention.

Table I
LDH ACTIVITY IN ELUTED FRACTIONS IN EXAMPLE 1.

Fraction No.	unspecific elution PVP-treatment		specific elution PVP-treatment	
	no	yes	no	yes
1	0.000	0.000	0.000	0.000
2	0.320	58.000	0.000	0.850
3	1.620	27.500	0.000	1.800
4	3.800	0.250	0.000	1.600
5	3.900	0.100	8.600	67.00
6	5.600		8.600	2.000
7	6.700		2.400	0.000
8	6.900		1.200	0.000
9	6.800		0.650	
10	6.600		0.500	
11	5.900		0.350	
12	5.400		0.250	
13	5.000		0.250	
14	4.500		0.200	
15	4.100		0.200	
16	3.700		0.200	
17	3.300		0.200	
18	2.900		0.150	
19	2.300		0.150	
20	2.100		0.100	

The unspecific elution of PVP untreated gel required collection of 35 more fractions to reach an LDH activity level of 0.150 U.

Table II

LDH PREPARATIONS PRODUCED BY DIFFERENT ELUTION MODES

Ligand	Mode of chromatography	Enzyme bound (U)	Enzyme eluted (U)	Elution Volume (ml)	Activity[a] (U/ml)	Protein[a] (mg/ml)	Specific activity[a] (U/ml)	Recovery (%)	Purification (-fold[a])
Cibacron Blue									
Untreated[b]	Non-specific elution	7120	5410	126.5	325	1.5	216	76	19
	Specific elution	12100	5590	110	499	2.1	237	46	20
PVP-treated[c]	Non-specific elution	4200	4040	11	1160	5.2	223	96	19
	Specific elution	3550	3480	13.8	777	3.8	204	98	18
Procion Scarlet									
Untreated[b]	Non-specific elution	1360	1280	10.8	760	13.8	55	94	4.7
	Specific elution	903	650	6.8	260	1.6	162	72	14
PVP-treated[c]	Non-specific elution	616	665	5.4	540	4.3	125	108	11
	Specific elution	375	364	5.4	290	1.6	180	97	15
Commercial sample					597	3.1	192		
Crude extract					64	5.5	11.8		

a) In the peak fraction

b) First run on the newly packed column

c) First run on the newly packed and PVP-treated column

Table III

SADH PREPARATIONS PRODUCED BY DIFFERENT ELUTION MODES

Ligand	Mode of chromatography	Enzyme bound (U)	Enzyme eluted (U)	Elution Volume (ml)	Activity[a] (U/ml)	Protein[a] (mg/ml)	Specific activity[a] (U/ml)	Recovery (%)	Purification (-fold[a])
Procion Scarlet									
Untreated[b]	Non-specific elution	1970	2130	8.1	308	6.1	50.5	108	14
	Specific elution	2525	1790	12.5	174	1.16	150	71	43
	Non-specific elution after specific elution		910	6.8	156	3.5	44.6	36	1
PVP-treated[c]	Non-specific elution	371	367	6.8	220	1.8	122	99	35
	Specific elution	240	224	8.1	81	0.5	162	93	46
	Non-specific elution after specific elution		19	6.8	7.8	0.6	13	8	3.7
Crude extract					49	14	3.5		

a) In the peak fraction

b) First run on the newly packed column

c) First run on the newly packed and PVP-treated column

Table IV
CIBACRON BLUE INTERACTION WITH VARIOUS PROTEINS AND PVP AND PVA.
DETERMINATION PRINCIPLE: DIFFERENCE SPECTROSCOPY.

Protein	Binding constant, μM	Ref.
SPECIFIC BINDING		
Dihydropholate reductase from chicken liver	0.8	1
Dihydropholate reductase from **Lactobacillus casei**	0.13	2
Phospholipase A_2	2	3
Cytochrome b_5 reductase	1	4
Nucleotide dehydrogenase rabbit muscle	0.5	6
Poly(ethylene imine)	0.1[a]	present work
PVCL	1.8	present work
PVP	2-6	present work
PVA	16.7	present work
NON-SPECIFIC BINDING		
Bovine serum albumin	35-85	7
Chitosan	50[b]	present work
Methyl cellulose	100[b]	present work
Cytochrome b_5 reductase	85	4

OTHERS: No binding could be detected for dextran, polyacrylamide and Eudragit®.

[a] The binding caused precipitation
[b] Estimated values

References

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C L A I M S

1. Method for affinity based isolation of a first target substance (T_1) from a mixture of substances containing said first target substance, said method comprising the steps of

(i) contacting said mixture in an aqueous medium with a matrix to which covalently is linked a ligand having binding affinity for said target substance (T_1) under conditions causing said target substance to bind to said ligand, and

(ii) changing the conditions offered by said medium so that said target substance (T_1) will elute from said matrix,

the method being **characterized** by the ligand having been initially complexed/bound to a second target substance (T_2) having weaker binding ability to the ligand than the first target substance (T_1) and providing multi-point attachment to the matrix by carrying several ligand binding sites and/or other matrix binding sites on its molecule enabling adsorption of the first target substance (T_1) during step (i) without the second target substance (T_2) becoming eluted from the matrix.

2. Method for affinity based isolation according to claim 1, **characterized** in that the second target substance (T_2) has 5 or more sites providing affinity for the ligand and/or other positions on the matrix.

3. Method for affinity based isolation according to claim 2, **characterized** in that the second target substance is a polymer, preferably a synthetic polymer, and in particular a homopolymer.

4. Method for affinity based isolation according to any of claims 1-3, **characterized** in

(a) that said second target substance (T_2) preferably is a polymer and is temperature-responsive in the sense that it in the medium used has a temperature limit (cloud

point) above which it becomes insoluble and below which it becomes soluble, and

(b) that steps (i) and (ii) are performed at different temperatures separated by the cloud point of the temperature-responsive polymer enabling elution of the first target substance (T_1) during step (ii).

5. Method for affinity based isolation according to claim 4, **characterized** in that the second target substance (T_2) is selected so that step (ii) is run below the cloud point.

6. Method for affinity based isolation according to any of claims 1-5, **characterized** in that said first target substance exhibits polypeptide structure.

7. Method for affinity based isolation according to any of claims 4-6, **characterized** in that said second target substance (T_2) is selected among those having cloud points that are below the temperature at which the first target substance T_1 becomes irreversibly damaged.

8. Method for affinity based isolation according to any of claims 1-7, **characterized** in that said ligand is a dye, in particular a triazine dye, such as Cibacron Blue or Procion Scarlet.

9. Method for affinity based isolation according to any of claims 1-18, **characterized** in that said second target substance (T_2) is poly(N-vinyl pyrrolidone) or poly(N-vinyl caprolactam).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 94/00016

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C07K 3/20, B01D 15/08, G01N 30/48
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C07K, G01N, B01D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A1, 0132948 (IQ (BIO)LIMITED), 13 February 1985 (13.02.85) --	1-9
A	EP, A1, 0056254 (WOOD DAVID ELDON), 21 July 1982 (21.07.82) -- --------	1-9

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 April 1994	19 -04- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT
Information on patent family members

26/02/94

International application No.
PCT/SE 94/00016

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0132948	13/02/85	SE-T3- 0132948	
		JP-A- 60036964	26/02/85
		US-A- 4668639	26/05/87
EP-A1- 0056254	21/07/82	US-A- 4716123	29/12/87

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